

REMARKS/ARGUMENTS

Claims 1-98 were previously pending. Claims 1-20, 23, 25-85 and 90-98 are withdrawn. Claims 21, 22, 24 and 86-89 have been examined. Claims 99 and 100 are added herewith. Support for the amendment to claim 21 is provided at, e.g., paragraph 363 (purified), paragraph 378 (modulation of angiogenesis), and paragraph 169 (indicating the zinc finger proteins and target sites in Tables 3 and 4 are merely exemplary). Support for the new claim 99 is provided at e.g., Table 3 (VOP32-E row). Support for new claim 100 is provided at, e.g., paragraph 147.

1-4. The Examiner maintains the restriction requirement on the basis that a search for all target sequences with each zinc finger protein listed in Tables 3 and 4 would be an undue burden. The Examiner says that applicants' election of VOP32-E limits the search to a single zinc finger protein with a single target sequence. Applicants request reconsideration.

It is respectfully submitted that the restriction requirement does not apply to any of claims 21-24, 86-89 or 100. These claims have been amended such that they no longer refer to specific sequences in Tables 3 and 4. Accordingly, these claims can be searched without reference to any particular target sequence in Tables 3 or 4, and should not therefore be restricted to a particular sequence. New claim 99 recites the amino acid segments of the three respective zinc fingers of VOP32E and therefore falls with the elected restriction group. Applicants note that the nature of the claimed subject matter is such that, in order to specify a single zinc finger protein, at least three sequences (*i.e.*, the sequence of the "recognition helix" region of each of three zinc fingers) must be specified. Thus, the restriction requirement is unduly burdensome in preventing Applicants from clearly and definitely claiming their invention.

5. The office action says that signed copies of four information disclosure statements (papers 7, 8, 12, and 13) are attached. However, these are missing from the office action actually received by applicants. Applicants request they be attached to the next communication from the PTO.

6. Claims 21-22, 24, 86-89 are objected to as reading on nonelected groups. As discussed above, applicants have requested the Examiner to modify the restriction requirement in

view of the amendment of the claims deleting reference to specific sequences from Tables 3 and 4 from all claims except claim 99. If the restriction requirement is so modified, then no further amendment of the claims is needed as the claims are directed to the elected sequence or are generic to it.

7-8. Claims 21-22 and 24 stand rejected as non-statutory subject matter. The Examiner says the claims should be amended to recite that the nucleic acids are isolated or purified. Applicants have amended the claims as suggested.

9. Claims 86-89 stand rejected under 35 USC 112, first paragraph for alleged lack of enablement. The Examiner acknowledges that the compositions are enabled for in vitro use. However, the Examiner takes the view that the claims are not enabled for in vivo use due to alleged unpredictability in using gene therapy techniques for treatment of tumors in humans. The Examiner takes the view that model systems are not predictive for testing the efficacy of drugs in humans. This rejection is respectfully traversed.

The office action focuses on alleged unpredictabilities in use of the claimed compositions to treat cancer in humans. However, an applicant "need only make one credible assertion of specific utility for the claimed invention to satisfy 35 U.S.C. 102 and 35 USC 112; additional statements of utility, even if not credible, do not render the invention lacking in utility" (MPEP 2107.02). Here, the Examiner has acknowledged that the compositions are enabled for in vitro use. This use alone satisfies the requirement of 35 USC 112, first paragraph for enablement. In addition, applicants note that the application provides examples showing the use of the claimed compositions to stimulate angiogenesis and wound healing in recognized animal models (see Example VI at pp. 88 et seq.) The Examiner has not disputed that this Example shows enablement of the compositions for stimulation of angiogenesis and wound healing in vivo. Given that the claimed compositions have several utilities for which enablement has been shown and has not been disputed, it is unnecessary to determine whether the claimed compositions are enabled for treatment of human cancers in vivo. Therefore, although applicants do not agree with the Examiner's remarks in this regard, they have not addressed them at this time. Withdrawal of the rejection is respectfully requested.

10-11. Claims 21 and 24 stand rejected under 35 USC 102(b) as anticipated by Choo. The Examiner acknowledges that Choo does not characterize a nucleic acid encoding a

protein that binds to a VEGF target sequence (examples of which are listed in Tables 3 and 4). However, the Examiner appears to take the view that such is inherent in Choo's zinc finger proteins. The Examiner also refers to teaching in the present specification (at p. 35) to the effect that multiple zinc finger proteins bind to the same target. Insofar as this rejection may be applied to the amended claims, it is respectfully traversed.

This and subsequent rejections appear to reflect a misunderstanding that any zinc finger protein can specifically bind to any target sequence. In fact, zinc finger proteins bind their target sequences in a sequence-specific manner (see e.g., Choo at col. 1, lines 29-62). Moreover, there are vast numbers of different potential target sequences and different zinc finger proteins. For example, for a target sequences of 9 nucleotides, there are 4^9 possible target sequences. For a single zinc finger, there are at least 5×10^8 permutations of residues that may affect binding specificity (see Choo at col. 14, lines 24-25). Given the large numbers of possible zinc fingers of different binding specificities, and the large number of possible target sites, the probability that a zinc_finger_protein_selected_at_random_binds_to_a_target_sequence_in_a_particular_gene_is vanishingly small.

Applicants' remarks at p. 35 of the specification are not inconsistent with the sequence-specificity of zinc finger proteins described above. This section of the specification indicates that a single zinc finger protein can bind to the same target sequence in multiple genes, or that multiple zinc finger proteins can bind to multiple target sequences in the same gene. A single zinc finger protein can bind to the same target sequence in multiple genes, because related genes (e.g., different VEGF genes) share segments of sequence identity and therefore contain the same target sequence. Multiple different zinc finger proteins can bind to multiple different target sequences in the same gene because a gene is much longer than a single target sequence. For example, a gene may be 100,000 bp or more in length; whereas a target sequence may be only 9 bp. Therefore, the statements at p. 35 of the specification are entirely consistent with the sequence-specific nature of zinc finger protein binding.

Turning now to the cited reference, Choo discusses selection of zinc finger protein libraries using the phage display technique. This technique permits the very rare zinc finger protein having a desired binding specificity to be selected from large libraries (col. 13, lines 53-56). The libraries selected by Choo contained a randomized central zinc finger flanked

on either side by fingers from the natural zinc finger protein, zif268 (see col. 13, lines 56-59). Choo also discusses two exemplary zinc finger proteins that specifically bind the underlined BCR-ABL target sequence shown in Fig. 6 and the ras target sequence shown at col. 32, lines 25-26 respectively.

The two genes regulated by Choo's exemplary zinc finger proteins are the BCR-ABL and ras genes respectively. These genes are not known to be related to VEGF. Nor does Choo disclose that regulation of either BCR-ABL or ras would result in modulation of angiogenesis. Accordingly, the specific zinc finger proteins disclosed by Choo cannot reasonably be assumed to bind to a VEGF gene and modulate angiogenesis, as claimed.

The libraries of nucleic acids encoding zinc finger proteins discussed by Choo also do not anticipate claim 21. As amended, claim 21 requires that the claimed nucleic acid be in purified form. By contrast, the nucleic acid sequence libraries in Choo constitute millions of nucleic acids in a mixture. The individual members of such libraries are not in purified form, and thus do not anticipate claim 21 as amended.

Further, it cannot be established under principles of inherency that the library of Choo contains a nucleic acid encoding a zinc finger protein that binds to a target site in a VEGF gene, as required by claim 21. “Inherency ... *may not be established by probabilities or possibilities.*” *Mehl/Biophile v. Milgraum*, 52 USPQ2d 1303, 1305 (Fed. Cir. 1999) (emphasis supplied). “The mere fact that a certain thing *may* result from a given set of circumstances *is not sufficient* to establish inherency.” *In re Rijckaert*, 28 USPQ2d 1955 (Fed. Cir. 1993) (emphasis supplied). Here, Choo's library contained a randomized central zinc finger flanked on either side by fingers from the natural zinc finger protein, zif268 (see col. 13, lines 56-59). Therefore, no variants are present for the flanking fingers. Even for the randomized central finger, only 2.6×10^6 out of 5.4×10^8 possible permutations were present (see col. 14, lines 22-27). Therefore, Choo's library encoded only a small proportion of all possible permutations of amino acids affecting the binding specificity of a zinc finger protein. Thus, it cannot be established with certainty that the nucleic acids present in Choos's library encoded a zinc finger protein that binds to a target site in a VEGF gene to modulate angiogenesis, as required by the present claims. For this additional reason, Choo's library does not inherently anticipate the present claims.

New claim 99 is distinguished on further grounds. New claim 99 specifies particular amino acid segments for each of three zinc fingers. Choo does not disclose a zinc finger protein having three fingers containing these respective segments.

12. Claim 21 stands rejected under 35 USC 102(b) as anticipated by Feldmann. Feldmann is said to teach a nucleic acid encoding a zinc finger protein. The Examiner acknowledges that Feldmann's zinc finger protein does not binds a target sequence in a VEGF gene (examples of which are provided in Tables 3 and 4), as specified in claim 21. However, the Examiner appears to take the view that such is inherent. The Examiner cites *In re Best* and *Ex parte Gray* for the proposition that the burden of showing that the claimed subject matter does not possess the same properties as the prior art can be shifted to Applicants. This rejection is respectfully traversed, particularly as applied to the amended claims.

The cases cited by the Examiner do not support shifting the burden to the applicant unless the Examiner has first established that the claims are "*reasonably* considered as possessing the allegedly inherent characteristics" of the prior art (*In re Best*, 195 USPQ 430, 433 (CCPA 1977), emphasis supplied). Here, the cited reference does little more than establish the existence and amino acid sequence composition of a particular protein comprising two zinc fingers. Feldmann absolutely fails to disclose a zinc finger protein that binds to a target site in a VEGF gene and modulates angiogenesis, as specified in claim 21, and there is nothing in the sequence of Feldmann's zinc finger protein that makes its binding to a VEGF gene, so as to modulate angiogenesis, inherent. As discussed above, inherency cannot be based on probabilities or possibilities, particularly such remote ones. Therefore, it is submitted that the Examiner has not established a *prima facie* case of inherency that requires rebuttal evidence from applicants.

Nevertheless, applicants draw the Examiner's attention to evidence indicating that the protein discussed by Feldmann in fact specifically regulates genes other than VEGF. Vyas et al., *Genetics* 158, 563-572 (2001) (copy attached) provide further information regarding NRG2 (the zinc finger protein discussed by Feldmann). In particular, Vyas reports that NRG2 activated expression of SUC2 and DOG2 genes. These genes are not known to be related to VEGF. Therefore, the Examiner has no reasonable basis to conclude that NRG2 binds a target site in a VEGF gene and modulates angiogenesis, as claimed.

Further, it is noted that the cited reference provides only an amino acid sequence for NRG2, and does not disclose a corresponding nucleic acid. The present claims are directed to purified nucleic acids.

For all of these reasons, it is respectfully submitted that the cited reference does not anticipate claim 21 (or claims dependent there from) under principles of inherency, and the rejection should be withdrawn.

13. Claims 21, 24 and 86-89 stand rejected as anticipated under 35 USC 102(b) by Barbas. The Examiner acknowledges that Barbas does not specifically teach a nucleic acid encoding a protein that binds to a target sequence in a VEGF gene as claimed. However, the Examiner apparently views such a binding characteristic as being inherent. The Examiner again cites *In re Best* and *In re Gray* as supporting shifting the burden of proof to applicants. This rejection is respectfully traversed particularly as applied to the amended claims.

Barbas discusses a phage display technique similar to that disclosed by Choo.

Like Choo, Barbas prepared libraries of proteins having a randomized central finger flanked by first and second fingers from zif 268 (see sentence bridging cols. 11 and 12). This library was then selected against a target sequence shown at col. 22, line 21. The selection process isolated several zinc finger proteins whose central fingers bind to the triplets shown in Table 1 (at cols. 9 and 10).

The nucleic acids in Barbas' libraries do not anticipate the present claims for reasons similar to those described in connection with Choo. First, the nucleic acids in Barbas' libraries are not in purified form. Second, Barbas' libraries do not include all possible binding specificities of zinc finger proteins (for example, only the central finger in a three finger protein was randomized). Thus, it cannot be concluded, with the certainty required under principles of inherency, that Barbas' libraries contain a nucleic acid encoding a protein that specifically binds to a target site in a VEGF gene and modulates angiogenesis as specified in claim 21.

The nucleic acids encoding zinc finger proteins selected from Barbas' library are not reasonably expected to encode proteins that specifically bind to a target site in a VEGF gene and modulate angiogenesis. This conclusion arises because the target sequence used by Barbas to select the zinc finger proteins is not known to be part of a VEGF gene, and the zinc finger

proteins were not selected or screened by Barbas for modulation of angiogenesis. Therefore, Barbas' zinc finger proteins would not bind to VEGF and modulate angiogenesis unless by remote chance there is a coincidental similarity between the target sequence used by Barbas and a target sequence in a VEGF gene, and further, that a zinc finger protein selected by Barbas has sufficient affinity for a target site within a VEGF gene to permit modulation of angiogenesis. Inherency cannot be found from such remote possibilities.

New claim 99 is distinguished on additional reasons as discussed in connection with the Choo reference.

For these reasons, withdrawal of the rejection is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-326-2400.

Respectfully submitted,



Joe Liebeschuetz
Reg. No. 37,505

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 650-326-2400
Fax: 415-576-0300
JOL:klc
60072278 v1

Interaction of the Repressors Nrg1 and Nrg2 With the Snf1 Protein Kinase in *Saccharomyces cerevisiae*

Valmik K. Vyas,* Sergei Kuchin† and Marian Carlson†,*

*Integrated Program in Cellular Biology, Molecular Biology and Biophysical Studies and †Departments of Genetics and Development and Microbiology, Columbia University, New York, New York 10032

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ABSTRACT

The Snf1 protein kinase is essential for the transcription of glucose-repressed genes in *Saccharomyces cerevisiae*. We identified Nrg2 as a protein that interacts with Snf1 in the two-hybrid system. Nrg2 is a C₂H₂ zinc-finger protein that is homologous to Nrg1, a repressor of the glucose- and Snf1-regulated *STA1* (glucoamylase) gene. Snf1 also interacts with Nrg1 in the two-hybrid system and co-immunoprecipitates with both Nrg1 and Nrg2 from cell extracts. A LexA fusion to Nrg2 represses transcription from a promoter containing LexA binding sites, indicating that Nrg2 also functions as a repressor. An Nrg1 fusion to green fluorescent protein is localized to the nucleus, and this localization is not regulated by carbon source. Finally, we show that VP16 fusions to Nrg1 and Nrg2 allow low-level expression of *SUC2* in glucose-grown cells, and we present evidence that Nrg1 and Nrg2 contribute to glucose repression of the *DOG2* gene. These results suggest that Nrg1 and Nrg2 are direct or indirect targets of the Snf1 kinase and function in glucose repression of a subset of Snf1-regulated genes.

THE Snf1 protein kinase is highly conserved from yeast-to-plants-to-mammals (for reviews, see CANCEDO 1998; HARDIE *et al.* 1998). In *Saccharomyces cerevisiae*, Snf1 is a key component of the glucose signaling pathway and is essential for the transcription of many glucose-repressed genes in response to glucose limitation (CELENZA and CARLSON 1986). This kinase also has roles in metabolic regulation, glycogen accumulation, stress responses, meiosis and sporulation, invasive growth, life span, and aging (THOMPSON-JAEGER *et al.* 1991; HARDY *et al.* 1994; HONIGBERG and LEE 1998; ASHRAFI *et al.* 2000; CULLEN and SPRAGUE 2000). Its mammalian homolog, AMP-activated protein kinase, regulates metabolism and transcription in response to the cellular energy supply, and the plant homologs are thought to be involved in sugar regulation.

The role of the Snf1 kinase in transcriptional control has been characterized in some detail. The adaptation of yeast cells to growth on nonpreferred carbon sources is accompanied by major changes in transcriptional patterns, and Snf1 appears to act at many control points. Thus far, Snf1 has been shown to regulate the expression and function of both transcriptional repressors and activators in response to glucose availability (for review, see CARLSON 1999). Recent evidence also implicates Snf1 in direct regulation of the RNA polymerase II holoenzyme (KUCHIN *et al.* 2000).

One of the major mechanisms by which Snf1 regulates transcription is by regulating the function of the transcriptional repressor Mig1. Mig1 is a zinc-finger protein that binds to sites in the promoters of many glucose-repressed genes and recruits the global corepressor Ssn6(Cyc8)-Tup1 (NEHLIN and RONNE 1990; TREITEL and CARLSON 1995; TZAMARIAS and STRUHL 1995). Several lines of evidence indicate that Snf1 phosphorylates Mig1 in response to glucose limitation and thereby regulates its nuclear localization and inhibits its repressor function (DEVIT *et al.* 1997; OSTLING and RONNE 1998; TREITEL *et al.* 1998).

Snf1 also effects transcriptional control by regulating transcriptional activators. Snf1 regulates the phosphorylation and function of the Cys₆ zinc-cluster activators Sip4 and Cat8, which bind to the carbon source-responsive elements (CSRE) of gluconeogenic genes (LESAGE *et al.* 1996; RAHNER *et al.* 1996, 1999; RANDEZ-GIL *et al.* 1997; VINCENT and CARLSON 1998; RAHNER *et al.* 1999). Snf1 has been shown to interact physically with Sip4 (LESAGE *et al.* 1996; VINCENT and CARLSON 1998).

The two-hybrid system has been useful in detecting interactions of Snf1 with transcriptional activators and repressors. Sip4 was first identified by its two-hybrid interaction with Snf1 (YANG *et al.* 1992). Mig1 also interacts with Snf1 in two-hybrid assays (TREITEL *et al.* 1998), and in this case interaction was much stronger with a catalytically defective mutant Snf1 protein, Snf1K84R (CELENZA and CARLSON 1986), which bears a substitution of arginine for the conserved lysine in the ATP-binding site. Several Srb/mediator proteins associated

Corresponding author: Marian Carlson, Columbia University, 701 W. 168th St., HSC922, New York, NY 10032.
E-mail: mbc1@columbia.edu

TABLE 1
Strains used in this study

Strain ^a	Genotype ^b
MCY829	<i>MATα his3 lys2 ura3</i>
MCY2916	<i>MATα snf1Δ10 his3 leu2 lys2 ura3</i>
MCY2693	<i>MATα snf1-K84R his3 leu2 ura3</i>
MCY3647	<i>MATα his3 leu2 lys2 ura3</i>
MCY3912	<i>MATα ade2 his3 leu2 lys2 trp1 ura3</i>
MCY3913	<i>MATα snf1Δ10 ade2 his3 leu2 lys2 trp1 ura3</i>
MCY4516	<i>MATα snf1Δ10 nrg1Δ::kanMX6 ade2 his3 leu2 lys2 trp1 ura3</i>
MCY4521	<i>MATα nrg1Δ::kanMX6 ade2 his3 leu2 lys2 trp1 ura3</i>
MCY4523	<i>MATα nrg2Δ::kanMX6 ade2 his3 leu2 lys2 trp1 ura3</i>
MCY4525	<i>MATα ade2 his3 leu2 lys2 trp1 ura3</i>
MCY4527	<i>MATα snf1Δ10 nrg2Δ::kanMX6 ade2 his3 leu2 lys2 trp1 ura3</i>
MCY4529	<i>MATα snf1Δ10 ade2 his3 leu2 lys2 trp1 ura3</i>
MCY4531	<i>MATα NRG1-GFP(S65T)::kanMX6 snf1Δ10 his3 leu2 lys2 ura3</i>
MCY4536	<i>MATα NRG2-GFP(S65T)::kanMX6 snf1Δ10 his3 leu2 lys2 ura3</i>
MCY4548	<i>MATα snf1Δ10 nrg1Δ::kanMX6 nrg2Δ::His3MX6 ade2 his3 leu2 lys2 trp1 ura3</i>
MCY4549	<i>MATα nrg1Δ::kanMX6 nrg2Δ::His3MX6 ade2 his3 leu2 lys2 trp1 ura3</i>
FY250 ^c	<i>MATα his3 leu2Δ1 trp1Δ63 ura3</i>
HF7C ^d	<i>MATα ade2 gal4 his3 leu2 lys2 trp1-901 LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3 URA3::GAL4_{17-mut(x)}-CYC1_{TATA}-lacZ</i>
CTY10-5d ^e	<i>MATα gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1-901</i>

^a MCY strains and FY250 have the S288C genetic background.

^b Alleles are *ura3-52*, *his3Δ200*, *lys2-801*, *ade2-101*, *leu2-3,112*, and *trp1Δ1* except where otherwise noted.

^c Gift of F. Winston (Harvard Medical School).

^d FEILOTTER *et al.* (1994).

^e Gift of R. Sternblanz (SUNY, Stonybrook, NY).

with the RNA polymerase II holoenzyme have also been shown to interact with Snf1 (KUCHIN *et al.* 2000).

In an effort to identify new downstream targets of the Snf1 kinase, we performed a two-hybrid screen with the catalytically defective Snf1K84R. We recovered Nrg2, a zinc-finger protein that is a close homolog of the DNA-binding repressor protein Nrg1. Nrg1 functions in glucose repression of the *STA1* gene, which encodes one of the glucoamylase isozymes responsible for starch degradation in *S. cerevisiae* var. *diastaticus* (PARK *et al.* 1999). Furthermore, the Snf1 kinase is known to be required for derepression of *STA2* (KUCHIN *et al.* 1993), which is identical to *STA1* throughout the promoter and coding region. We therefore examined the relationship of Nrg1 and Nrg2 to the Snf1 kinase. Both proteins interact with Snf1 in the two-hybrid system and both co-immunoprecipitate with Snf1 from cell extracts. We present evidence that Nrg2 functions as a repressor and that the nuclear localization of Nrg1, unlike that of Mig1, is not regulated by carbon source. Finally, we examine the roles of Nrg1 and Nrg2 in repression of Snf1-dependent genes.

MATERIALS AND METHODS

Strains and genetic methods: The *S. cerevisiae* strains used are listed in Table 1. To construct strain MCY4531, we first generated a 2.5-kb DNA fragment using the polymerase chain reaction (PCR) with the template pFA6a-GFP(S65T)-kanMX6 (LONGTINE *et al.* 1998) and two primers, one including the

15 codons preceding the termination codon of *NRG1* and the other including 45 base pairs following the stop codon. This fragment was used to transform strain MCY2916, with selection on rich medium containing 200 µg/ml G418 (Life Technologies, Gaithersburg, MD), and its integration at the *NRG1* locus was verified by PCR. Strain MCY4536 was created in the same way, except that the targeting sequence was for *NRG2*. To construct the *nrg1Δ::kanMX6*, *nrg2Δ::kanMX6*, and *nrg2Δ::His3MX6* mutations, we first amplified *kanMX6* or *His3MX6* (LONGTINE *et al.* 1998) with oligonucleotides containing 45 nucleotides flanking the open reading frame of *NRG1* or *NRG2*. PCR products were used to transform MCY3912 or MCY3913 with selection for the marker, and disruption was verified by PCR amplification of genomic DNA.

Standard methods for yeast genetic analysis and transformation were used (ROSE *et al.* 1990). Cells were grown in synthetic complete (SC) medium lacking appropriate supplements to maintain selection for plasmids.

Plasmids: Plasmids used in this study are listed in Table 2. pRJ215 contains the *Bam*HI fragment from pRJ80 (LUDIN *et al.* 1998) in the *Bam*HI site of pEG202 (GOLEMIS *et al.* 1997). pSK106 was created by cloning the *Bam*HI fragment from pRJ215 into the *Bam*HI site of pGBT-9 (BARTEL *et al.* 1993). The coding regions of *NRG1* and *NRG2* were amplified by PCR from genomic DNA using Vent polymerase (New England Biolabs, Beverly, MA) with oligonucleotides K47 and K48 (Nrg2) and K49 and K50 (Nrg1), each containing a *Bam*HI site. *Bam*HI-digested PCR product was cloned into the *Bam*HI site of plasmids pACTII (LEGRAIN *et al.* 1994), pVP16 (VOJTEK *et al.* 1993), pWS93 (SONG and CARLSON 1998), pSH2-1 (HANES and BRENT 1989), and pEG202.

Oligonucleotides: Oligonucleotides used as primers in PCR were the following: K47, GCGCCGGATCCTAATGTCCATAGG TTACAAAGAC; K48, GCGCCGGATCCTCAACTGCTAGCCT CCCTC; K49, GCGCCGGATCCTAATGTTTACCCATATAA

TABLE 2
Expression plasmids used in this study

Name	Vector	Expressed protein	Source or reference
pSK106	pGBT-9	GBD-Snf1K84R	This study
pRJ190	pSH2-1	LexA _{S7} -Snf1KD	JIANG and CARLSON (1996)
pRJ192	pSH2-1	LexA _{K7} -Snf1RD	R. JIANG and M. CARLSON, unpublished data
pV37	pSH2-1	LexA _{S7} -Nrg2	This study
pV38	pSH2-1	LexA _{S7} -Nrg1	This study
pV39	pACTII	GAD-Nrg2	This study
pV40	pACTII	GAD-Nrg1	This study
pV45	pVP16	VP16-Nrg2	This study
pV46	pVP16	VP16-Nrg1	This study
pV35	pWS93	HA _{S7} -Nrg2	This study
pV36	pWS93	HA _{S7} -Nrg1	This study
pSK117	pSK37	Snf1	TREITEL <i>et al.</i> (1998)
pSK118	pSK37	Snf1K84R	TREITEL <i>et al.</i> (1998)
pIT469	pEG202	LexA-Snf1	KUCHIN <i>et al.</i> (2000)
pRJ215	pEG202	LexA-Snf1K84R	R. JIANG and M. CARLSON, unpublished data

CTATAG; K50, CCGCGGATCCGTCAATTATTGTCCCTT TTC (*Bam*H sites are underlined).

Two-hybrid screen: A two-hybrid screen (FIELDS and SONG 1989) for proteins that interact with a Gal4 DNA-binding domain (CBD) fusion to Snf1K84R was carried out in strain HF7C, which contains a chromosomally located *GAL1-HIS3* reporter. The strain was transformed with pSK106, which expresses GBD-Snf1K84R, and with a library (gift of S. Elledge, Baylor College of Medicine) of *S. cerevisiae* cDNAs fused to the Gal4 activating domain (GAD). Transformants were selected on SC-His + 2% glucose plates for a His⁺ phenotype. Five plasmids conferred a His⁺ phenotype and gave blue color in CTY10-5d and were subjected to sequence analysis. Besides *NRG2*, the recovered sequences were *SNF4*, *CPM1*, *POR1*, and *TDH1*. The others were not analyzed further.

Two-hybrid assays with LexA fusion proteins were carried out in strain CTY10-5d or in strain FY250 transformed with the pSH18-34 reporter, containing LexA binding sites 5' to a *GAL1-lacZ* reporter (ESTOJAK *et al.* 1995; GOLEMIS *et al.* 1997).

Invertase and β-galactosidase assays: Invertase activity was assayed in whole cells as previously described (JIANG and CARLSON 1996). β-Galactosidase activity was assayed in permeabilized cells (GUARENTE 1983) and expressed in Miller units (MILLER 1972). A filter assay in which β-galactosidase activity confers blue color was also used as previously described (JIANG and CARLSON 1996).

Co-immunoprecipitation assays: Preparation of protein extracts and immunoprecipitation procedures were essentially as described previously (CELENZA *et al.* 1989). The extraction buffer was 50 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM dithiothreitol, 10% glycerol, and contained 2 mM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Roche Molecular Biochemical). Proteins were immunoprecipitated with anti(α)-hemagglutinin (HA) monoclonal antibody (Roche Molecular Biochemical) or α-LexA monoclonal antibody (CLONTECH, Palo Alto, CA) in the same buffer, except that it contained 0.25% Triton X-100 for immunoprecipitation with α-HA and 50 mM NaCl and 0.1% Triton X-100 for immunoprecipitation with α-LexA.

Immunoblot analysis: Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting using polyclonal α-Snf1 (CELENZA and CARLSON 1986), monoclonal α-HA (Roche Molecular Biochemical), or polyclonal α-LexA (In-

vitrogen, San Diego). Antibodies were detected by chemiluminescence with ECL or ECL Plus reagents (Amersham, Arlington Heights, IL).

Imaging of green fluorescent protein fluorescence: Cells were grown to midlog phase in synthetic media containing 5% glucose or 2% glycerol plus 2% ethanol, harvested by centrifugation, and resuspended in nonfluorescent media [0.9 g/liter-KH₂PO₄, 0.23 g/liter-K₂HPO₄, 0.5 g/liter-MgSO₄, 3.5 g/liter-(NH₄)₂SO₄] containing the appropriate carbon source. Nuclei were stained by addition of 0.8 μg/ml of 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Fluorescence of green fluorescent protein (GFP) fusion proteins was visualized in unfixed cells by using a Nikon Eclipse E800 fluorescent microscope. Images were captured by using a digital camera (Hamamatsu Orca-100, Hamamatsu, Japan) and Openlab software (Improvision) and were converted to Adobe Photoshop 2.5.1 files for processing.

Microarray analysis: Strain MCY3912 carrying pV46 or pVP16 was grown in SC-Leu + 5% glucose. Total yeast RNA was extracted with hot phenol, and poly(A)⁺ RNA was purified by oligo(dT) chromatography (QIAGEN, Chatsworth, CA). Fluorescently labeled cDNA was prepared, and expression of 5805 yeast open reading frames (ORFs) was analyzed using DNA microarrays as described (DRISI *et al.* 1997). Microarrays were produced by the Columbia University Microarray Project.

RESULTS

Identification of Nrg2 in a two-hybrid screen for interaction with Snf1: We carried out a two-hybrid screen for proteins that interact with the catalytically defective Snf1 protein kinase, Snf1K84R. GBD-Snf1K84R was used as a bait to screen a library of cDNAs fused to GAD. We recovered six clones that were His⁺ in combination with GBD-Snf1K84R (see MATERIALS AND METHODS). Five clones also caused blue color in combination with both LexA-Snf1K84R and LexA-Snf1 in strain CTY10-5d.

Sequence analysis showed that one of these clones (pKRIP6) encodes an in-frame fusion of GAD at a position five nucleotides preceding codon 1 of the *NRG2*

Nrg1	1	M F Y P Y N Y S N L N V S T M P A L P G I S - - - A F D G M Q D E - - - E N V E I S P E R K Y Q
Nrg2	1	M S I G Y K - D N E M S T I L A K D R K C E F F P I N E C S P S Q I T L M P E M F S F N N E R K Y Q
Nrg1	43	T L P P V E T N S H Y V V E N E L K E K L N K T A F D E R Z Q T K S E N G S E K W E P K Y L I T P N L
Nrg2	50	T L P P L E K T S H G T D D U L K D K L N K C A F D F - E S G K Q A N - - - - -
Nrg1	93	Q T R E V S F D N S E V Q Y N S D S S E K S S G S Q L T C N S S I I Q Q P E N G I V S N D A Y N K M
Nrg2	84	- - R T - - - S D G R E S R L C A S G K T S P E L P L Q - N I N I L K A E N T C N G K S D P Y S S I
Nrg1	143	A N S R Y S L K T R I Q R T D P R N T L E D E E D L E Q R R K Y I C K I C A R G F T T S G H L A R H
Nrg2	128	K I S - - - K P T K T V I K L K S T K T N T A G -- Q R T R H F K C I C S T G F T T S G H L S R H
Nrg1	193	* * * * * N R I H T G E K N H C C P Y K G C I Q R F S R H D N C L Q H Y R T H L K N G Q - - - - -
Nrg2	172	N R I H T G E K N H I C P H E G C C Q R F S R H D N C L Q H Y R T H A N K K R N W K R R E A S S

sults); both are part of the Biology Workbench v3.2 package (<http://bohm.psu.edu/BiologyWorkbench/>). C₂H₂ zinc fingers are marked with asterisks (BOHM *et al.* 1997).

gene (YBR066C). *NRG2* encodes a protein of 220 amino acids with a predicted molecular mass of 25 kD and two zinc fingers at the C terminus. The zinc fingers are homologous to those of the transcriptional activators Msn2 and Msn4, which bind stress response elements (MARTINEZ-PASTOR *et al.* 1996), and to those of the repressors Mig1 and Mig2. However, the closest homolog to *Nrg2* is *Nrg1*, which is 44% similar overall and 84% identical in the zinc-finger region (Figure 1).

NRG1 was identified as a multicopy inhibitor of the glucose-repressible *STA1* promoter in *S. cerevisiae* var. *diastaticus* (PARK *et al.* 1999). In the selection scheme, the *STA1* promoter was used to drive expression of *TPK2*, encoding a catalytic subunit of cyclic AMP-dependent protein kinase, which is toxic to cells at high levels. Multicopy *NRG1* restored healthy growth and repressed glucoamylase expression. Nrg1 bound to sites in the *STA1* upstream region, and a LexA fusion to Nrg1 repressed transcription of a reporter with LexA sites, dependent on the corepressor Ssn6-Tup1 (PARK *et al.* 1999). Mutation of *NRG1* relieved glucose repression of *STA1*. Release from glucose repression of glucoamylase genes requires the Snf1 protein kinase (KUCHIN *et al.* 1993), and thus these studies suggest a functional connection between Nrg1 and Snf1. We therefore included Nrg1 in subsequent experiments to assess its interaction with Snf1.

The Snf1 kinase comprises a catalytic domain (residues 1–392, designated Snf1KD) and a regulatory domain (residues 392–633, designated Snf1RD), which binds to the kinase domain and inhibits its activity (JIANG and CARLSON 1996). We assayed the two-hybrid interaction of GAD-Nrg1 and GAD-Nrg2 with LexA DNA-binding domain (LexA₄₇) fusions to Snf1KD and Snf1RD. Interaction was monitored by assaying β-galactosidase activity in cells grown under glucose-repressing conditions (5% glucose) and after a 3-hr shift to 0.05% glucose. Both GAD-Nrg1 and GAD-Nrg2 interacted with LexA₄₇-Snf1KD but not with LexA₄₇-Snf1RD (Table 3).

Co-immunoprecipitation of the Snf1 kinase with Nrg1 and Nrg2: To confirm that Snf1 interacts with Nrg1 and Nrg2, we tested for co-immunoprecipitation of the

FIGURE 1.—Alignment of Nrg1 and Nrg2. Protein sequences are indicated in single letter code, and numbers indicate amino acid residues. Solid boxes indicate identities, shaded boxes indicate similarities, and dashes indicate gaps in the alignment. Alignment was done using the programs MSA (GUPTA *et al.* 1995; KECECIOGLU *et al.* 1995; LIPMAN *et al.* 1989) and Boxshade (K. HOFMANN and M. D. BARON, unpublished re-

kinase with triple HA epitope-tagged proteins. Protein extracts were prepared from *snf1*^A cells expressing HA-Nrg1 or HA-Nrg2 and Snf1, Snf1K84R, or no Snf1 protein. Immunoblot analysis showed that levels of both HA-Nrg1 and HA-Nrg2 were severely reduced in cells expressing no Snf1 protein, but levels were normal in cells expressing Snf1K84R and thus defective only for Snf1 catalytic activity (Figure 2A; input panel); these results are consistent with physical interactions *in vivo*. Proteins were immunoprecipitated with α -HA antibodies, and the precipitates were analyzed by SDS-PAGE and immunoblot analysis with α -Snf1 antibodies. Snf1 and Snf1K84R co-immunoprecipitated with both HA-Nrg1 and HA-Nrg2, but coprecipitation with HA-Nrg2 was more efficient (Figure 2A; CoIP panel). In control experiments, Snf1 did not coprecipitate with the triple HA tag expressed from the vector.

We also tested for co-immunoprecipitation of HA-Nrg1 and HA-Nrg2 with LexA-Snfl (Figure 2B). Extracts were prepared from wild-type cells expressing LexA-

TABLE 3
Nrg1 and Nrg2 interact with the Snf1 catalytic domain
in the two-hybrid assay

LexA fusion	GAD fusion	5% glucose	Shift
LexA ₈₇ -Snf1 KD	GAD-Nrg1	2.6	11
	GAD-Nrg2	12	13
	GAD	0.7	3.1
LexA ₈₇ -Snf1 RD	GAD-Nrg1	0.5	0.3
	GAD-Nrg2	0.6	0.3
	GAD	0.2	0.1

Transformants of strain FY250 expressed the indicated fusion proteins from plasmids listed in Table 2 and carried the *lacZ* reporter plasmid pSH18-34. Transformants were grown in selective SC + 5% glucose medium (5% glucose) and shifted to SC + 0.05% glucose for 3 hr (Shift). Values are the average β -galactosidase activity for 5–15 transformants. Standard errors were <15%. Additional control experiments showed that strains expressing LexA₈₇ in combination with each of the GAD proteins produced no significant β -galactosidase activity.

Snf1 or LexA-Snf1K84R and HA-Nrg1 or HA-Nrg2, and LexA proteins were immunoprecipitated with α -LexA antibodies. Immunoblot analysis with α -HA antibody showed that HA-Nrg1 and HA-Nrg2 co-immunoprecipitated with both LexA-Snf1 and LexA-Snf1K84R, but not with LexA alone. Again, HA-Nrg1 was less efficiently recovered than HA-Nrg2.

To examine the possibility that Snf1 phosphorylates Nrg1 or Nrg2, we performed kinase assays with both of the above sets of immunoprecipitates. No Snf1-dependent phosphorylation of Nrg1 or Nrg2 was detected (data not shown). In addition, we examined HA-Nrg1 and HA-Nrg2 for phosphorylation *in vivo* by immunoblot analysis. No Snf1-dependent differences in the mobility of either protein were detected when wild-type and *snf1* mutant cells were grown in 5% glucose or shifted to 0.05% glucose or shifted to 2% glycerol plus 2% ethanol (data not shown). Subsequent analysis of

LexA fusion proteins also revealed no Snf1-dependent differences in mobility (Figure 3C).

Repressor function of Nrg1 and Nrg2: Previous studies showed that LexA₈₇-Nrg1 represses expression of a reporter with LexA binding sites in glucose-grown cells (PARK *et al.* 1999). We therefore tested the ability of LexA₈₇-Nrg2 to function as a repressor in this assay. Wild-type strain MCY829 was transformed with plasmids expressing LexA₈₇-Nrg1, LexA₈₇-Nrg2, or LexA₈₇ alone from the *ADH1* promoter, and a *CYC1-lacZ* reporter with either four or zero LexA binding sites 5' to the upstream activation sites (UAS; Figure 3A). Reporter gene expression was monitored by assaying β -galactosidase activity. LexA₈₇-Nrg1 and LexA₈₇-Nrg2 both repressed transcription about 15-fold in cells grown in 5% glucose (Figure 3B).

We then examined the regulation of transcriptional repression by carbon source. Repression by both LexA₈₇-Nrg1 and LexA₈₇-Nrg2 was maintained after a shift to low glucose (0.05%) or during steady-state growth in raffinose, sucrose, or galactose (data not shown). In accord with these findings, the *STA* genes are not derepressed under these growth conditions, but only during growth in glycerol plus ethanol (PRETORIUS *et al.* 1986; KARTASHEVA *et al.* 1996). No repression of the reporter was observed when cells were grown in 2% glycerol plus 2% ethanol (Figure 3B); however, immunoblot analysis showed that levels of both fusion proteins were very low when cells were grown in glycerol plus ethanol (Figure 3C), which could account for the lack of repression. The low protein levels can probably be attributed to reduced expression from the *ADH1* promoter because levels of *ADH1* mRNA are 6- to 10-fold lower during growth on ethanol than on glucose (DENIS *et al.* 1983). Moreover, the levels of HA-Nrg1, HA-Nrg2, and LexA₈₇-Sip4, when expressed from this vector, were also very low in glycerol/ethanol-grown cells (data not shown). These data exclude effects specific to the LexA₈₇ tag.

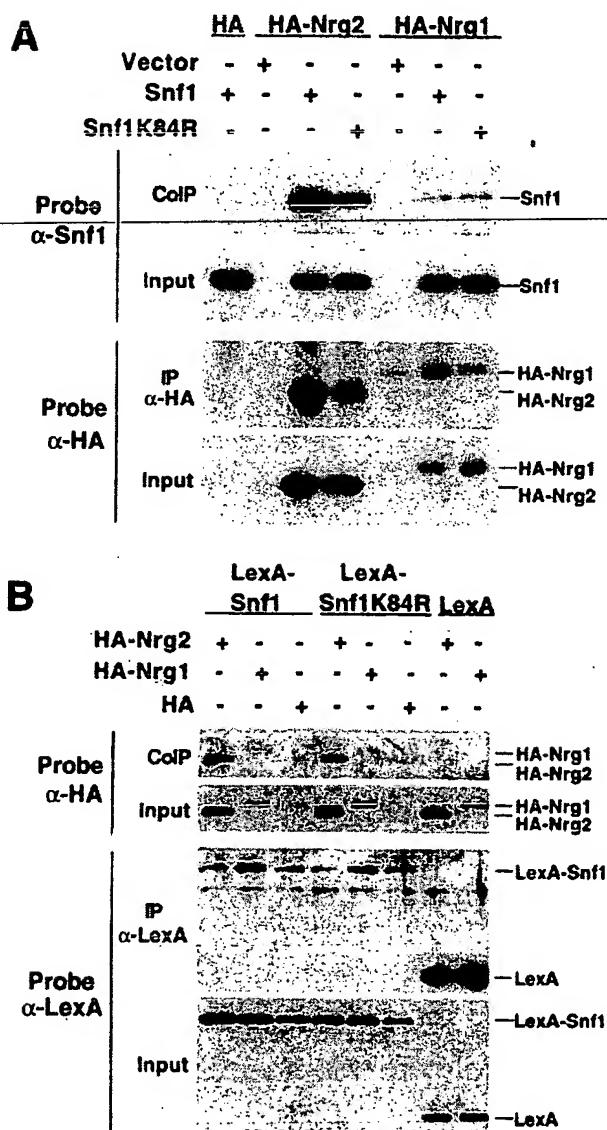


FIGURE 2.—Co-immunoprecipitation of Snf1 with Nrg1 and Nrg2. (A) Strain MCY2916 (*snf1*Δ) was transformed with a plasmid expressing HA-Nrg1 or HA-Nrg2 or the parent vector pWS93 expressing the triple HA epitope and also with a plasmid expressing Snf1 or Snf1K84R or the vector pSK37 (see Table 2). Cells were grown in selective medium containing 5% glucose. Protein extracts (50 μ g) were immunoprecipitated (IP) with α -HA antibodies. Input extracts (10 μ g) and precipitates were resolved by 12% SDS-PAGE, blotted, and immunodetected with α -Snf1. The filter was stripped and reprobed with α -HA to detect the HA fusion protein. (B) Strain MCY3647 was transformed with plasmids expressing either LexA, LexA-Snf1, or LexA-Snf1K84R and HA-Nrg1, HA-Nrg2, or HA alone. Cells were grown in selective medium containing 2% glucose. Protein extracts (50 μ g) were immunoprecipitated with monoclonal α -LexA antibodies. Precipitates and input extracts (10 μ g) were resolved by 12% SDS-PAGE, blotted, and immunodetected with α -HA. The filter was then stripped and reprobed with polyclonal α -LexA.

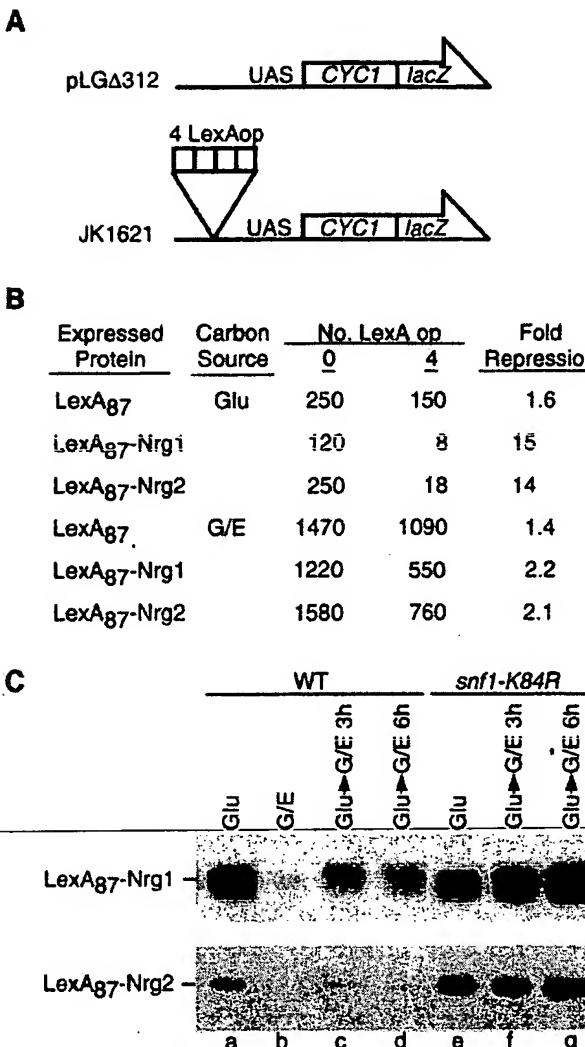


FIGURE 3.—Transcriptional repression by LexA₈₇-Nrg1 and LexA₈₇-Nrg2. (A) Reporter plasmids, pLGΔ312 contains the *lacZ* gene under the control of the *CYC1* promoter and UAS (GUARANTE and HOAR 1984). JK1621 is derived from pLGΔ312 and contains four lexA operators (op) 5' to the UAS (KELEHER *et al.* 1992). (B) Repression of reporter gene expression by the indicated LexA protein in wild-type strain MCY829. Transformants were grown selectively to midlogarithmic phase in 5% glucose (Glu) or in 2% glycerol plus 2% ethanol (G/E). β -Galactosidase activity was assayed in permeabilized cells and expressed in Miller units. Values represent the mean of five transformants and have a standard error of <15%. (C) Wild-type (WT) strain MCY829 (lanes a-d) and the *snf1-K84R* mutant MCY2693 (lanes e-g) were transformed with JK1621 and a plasmid expressing either LexA₈₇-Nrg1 (top) or LexA₈₇-Nrg2 (bottom). Transformants were grown in Glu or G/E, as above, or shifted from Glu to G/E for 3 or 6 hr, as indicated. Protein extracts were prepared by a rapid boiling method (VINCENT and CARLSON 1999), resolved by 10% SDS-PAGE, and immunoblotted with polyclonal α -LexA. For each LexA protein, all lanes shown are from the same exposure of the same immunoblot. No degradation products were detected. Analysis of a second set of transformants gave the same results.

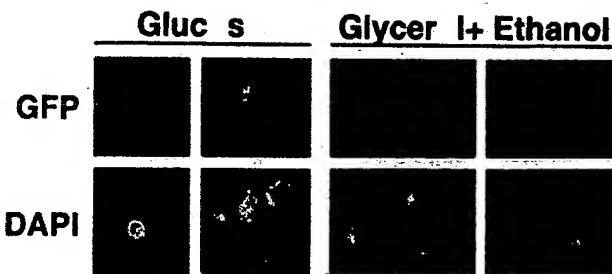


FIGURE 4.—Nuclear localization of Nrg1-GFP. Strain MCY4531 was transformed with plasmid pSK117 to provide Snf1 function, and cells were grown in selective synthetic medium containing 5% glucose or 2% glycerol plus 2% ethanol and stained with DAPI. GFP and DAPI fluorescence were visualized as described in MATERIALS AND METHODS.

and Sip4 is unlikely to be specifically degraded under these conditions as it functions in the activation of gluconeogenic genes. PARK *et al.* (1999) reported that LexA₈₇-Nrg1 does not repress in cells grown in glycerol plus ethanol, but it is likely that the protein was not present because their expression vector was identical to ours.

We attempted to obtain evidence for a role of Snf1 in inhibiting repressor function by examining release from repression in wild-type and *snf1* mutant cells after a shift from glucose to glycerol plus ethanol. Unfortunately, in wild-type cells the release occurred too slowly. Cells expressing LexA₈₇-Nrg1 or LexA₈₇-Nrg2 and containing a reporter with LexA sites only doubled their β -galactosidase activity during a 3-hr shift (repression ratios decreased to ~7; data not shown), and protein levels were already lower by 3 hr (Figure 3C). In *snf1-K84R* mutant cells, LexA₈₇-Nrg1 and LexA₈₇-Nrg2 repressed transcription ~15-fold during growth on glucose, and repression was not relieved during the 3-hr shift (repression ratios of ~20; data not shown); however, protein levels remained high in the mutant cells (Figure 3C).

Nuclear localization of Nrg1: One of the mechanisms by which the Snf1 kinase regulates the function of the Mig1 repressor in response to the glucose signal is by regulating its nuclear localization (DEVIT *et al.* 1997; DEVIT and JOHNSTON 1999). To address the possibility that the localization of Nrg1 or Nrg2 is similarly regulated, we constructed strains expressing Nrg1 or Nrg2 fused to GFP from the cognate chromosomal promoters. We did not detect significant fluorescence from Nrg2-GFP. Nrg1-GFP was localized in the nucleus when cells were grown in glucose, consistent with its function as a repressor, but also remained in the nucleus when cells were grown in glycerol plus ethanol (Figure 4). Similarly, no export of Nrg1-GFP was observed when cells were shifted from glucose to glycerol for 15 min (data not shown). Immunoblot analysis confirmed that Nrg1-GFP is a stable protein (data not shown). These findings suggest that the ability of Nrg1 to function as

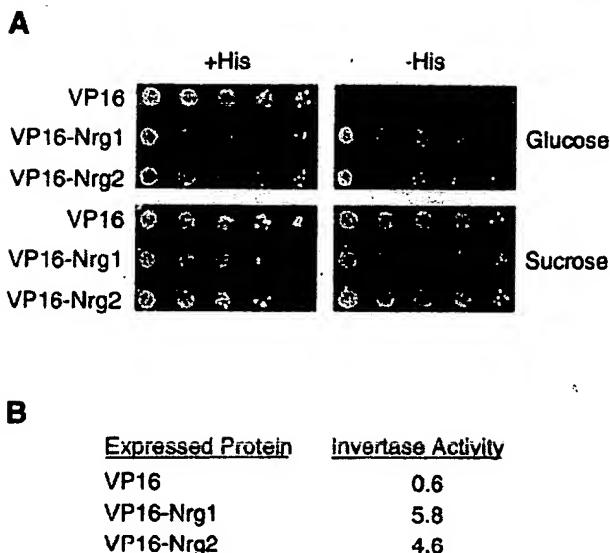


FIGURE 5.—VP16-Nrg1 and VP16-Nrg2 relieve glucose repression of the *SUC2* promoter. (A) Strain MCY3912 (*his3*) was transformed with the *CEN-TRP1* plasmid pYSH bearing the *SUC2::HIS3* reporter (TU and CARLSON 1994) and plasmids expressing VP16, VP16-Nrg1, or VP16-Nrg2 (see Table 2). Serial sevenfold dilutions of cell suspensions were spotted on SC-Trp-Leu or SC-Trp-Leu-His containing 4% glucose or 2% sucrose, as indicated. The plates were photographed after 4 days at 30°. (B) Expression of the native *SUC2* gene was monitored by invertase assays of strain MCY3912 transformed with the same expression plasmids. Values represent the mean for five transformants, with a standard error of <15%.

a repressor is not regulated by differential subcellular localization.

Nrg1 and Nrg2 fused to the VP16 activation domain activate low-level *SUC2* expression in glucose: The repressor Mig1 has broad roles in glucose repression of many genes, whereas thus far, Nrg1 is known to affect only the glucoamylase genes. *NRG1* and *NRG2* have not been identified genetically in searches for regulators of other glucose-repressed genes; however, it is possible that their contributions to repression are modest. To test the possibility that Nrg1 and Nrg2 regulate *SUC2*, another glucose-repressed gene that is controlled by the Snf1 pathway, we fused the viral VP16 activation domain to both Nrg proteins. Our rationale was that the overexpressed VP16-Nrg fusion protein would compete with the native protein for binding to its sites. Similar fusions of VP16 to the repressor Mig1 activate expression of *SUC2* in glucose-grown cells (OSTLING *et al.* 1996).

We first used a *SUC2::HIS3* reporter that exhibits glucose-repressible expression of *HIS3* (TU and CARLSON 1994). Transformants of strain MCY3912 (*his3*) bearing *SUC2::HIS3* and expressing VP16-Nrg1 or VP16-Nrg2 were His⁺ on medium containing 2% glucose, whereas transformants expressing VP16 alone were His⁻ (Figure 5A). All transformants were His⁺ on medium containing 2% sucrose. We also assayed expression of invertase

activity from the chromosomal *SUC2* locus in cells grown in 5% glucose. VP16-Nrg1 and VP16-Nrg2 both activated *SUC2* expression about 10-fold relative to VP16 alone (Figure 5B). However, invertase activity was still about 30-fold lower than the levels typically detected in derepressed wild-type cells. Although it is possible that overexpression of the fusion proteins leads to aberrant binding, these data suggest that the native Nrg1 and Nrg2 proteins have some role in glucose repression of *SUC2*, which may involve either direct binding to the *SUC2* promoter or an indirect mechanism. Consistent with these results, an independent study has demonstrated that an *nrg1Δ* mutation causes defects in glucose repression of transcription of the *SUC2*, *GAL1*, and *GAL10* genes (ZHOU and WINSTON 2001).

Mutations in *NRG1* and *NRG2* affect glucose repression of the *DOG2* gene: To identify other Snf1-dependent genes that are targets of repression, we used DNA microarray analysis to identify genes that are upregulated in glucose-grown cells expressing VP16-Nrg1, as compared to cells expressing VP16 alone (data not shown; see MATERIALS AND METHODS). Among the genes that were upregulated, we identified one gene, *DOG2*, that is known to be regulated by glucose repression and by Snf1 (RANDEZ-GIL *et al.* 1995; LUTFIYYA *et al.* 1998; TSUJIMOTO *et al.* 2000). *DOG2* encodes 2-deoxyglucose-6-phosphate phosphatase and confers resistance to 2-deoxyglucose toxicity. *DOG2* is regulated by the repressors Mig1 and Mig2 in response to glucose and also by the stress response factors Msn2 and Msn4.

To explore the regulation of *DOG2* by Nrg1 and Nrg2, we constructed *nrg1Δ* and *nrg2Δ* single and double mutants (see MATERIALS AND METHODS) and introduced a plasmid bearing a *DOG2* promoter fusion to *lacZ*, pBM3501 (LUTFIYYA *et al.* 1998). When transformants were grown in 2% glucose, the mutants showed a two- to threefold elevation in β-galactosidase activity (2.1–3.3 units) relative to wild type (1.1 units); values are averages for 5–15 transformants. Although the defect in glucose repression is small, Nrg1 and Nrg2 are predicted to have only a modest role. Mig1 and Mig2 are largely responsible for glucose repression of *DOG2-lacZ*, and the residual repression in a *mig1Δ mig2Δ* double mutant is only twofold (LUTFIYYA *et al.* 1998). The *nrg1Δ* and *nrg2Δ* mutations did not affect derepression as all strains produced similar activity (15–20 units) after a shift to 0.05% glucose for 3 hr.

Previous studies showed that derepression of *DOG2* in response to glucose limitation requires the Snf1 kinase (TSUJIMOTO *et al.* 2000). To examine the relationship of Snf1 to Nrg1 and Nrg2, we assayed derepression of *DOG2-lacZ* in strains mutant for *snf1Δ* in combination with *nrg1Δ* or *nrg2Δ* or both. Transformants were grown in 2% glucose, shifted to 0.05% glucose for 3 hr, and assayed for β-galactosidase activity. Both *nrg1Δ* and *nrg2Δ* partially suppressed the *snf1Δ* mutant defect (Fig-

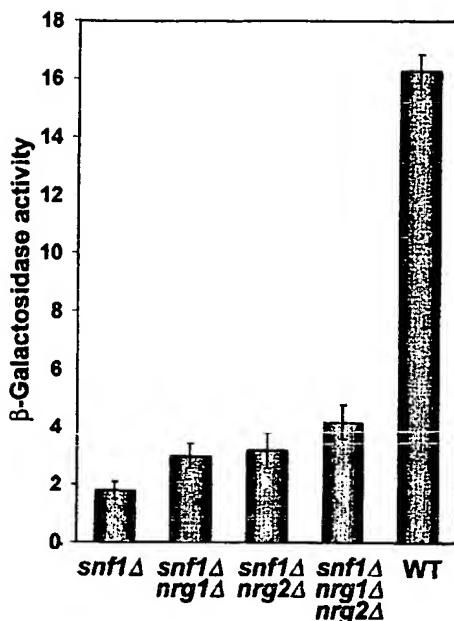


FIGURE 6.—Suppression of the *snf1* mutant defect in derepression of *DOG2-lacZ* by *nrg1* and *nrg2* mutations. Strains were MCY4529, MCY4516, MCY4527, and MCY4548 transformed with the *DOG2-lacZ* reporter plasmid pBM3501 (LUTFIYYA *et al.* 1998). Cultures were grown to midlog phase in SC-Ura + 2% glucose and then shifted to SC-Ura + 0.05% glucose for 3 hr. β -Galactosidase was assayed in permeabilized cells, and values are averages for 10–15 transformants.

ure 6). Again the effect is small, but only partial suppression would be expected because repression by Mig1 is not relieved in a *snf1* Δ mutant (DEVIT *et al.* 1997; OSTLING and RONNE 1998; TREITEL *et al.* 1998). These findings suggest that one of the roles of Snf1 in regulating the *DOG2* promoter is to inhibit repression by Nrg1 and Nrg2.

DISCUSSION

We have identified Nrg2 in a two-hybrid screen for proteins that interact with the Snf1 protein kinase. Nrg2 was of particular interest because its close homolog, Nrg1, functions in glucose repression of the *STA1* gene, and release from repression depends on Snf1. We here present evidence that both Nrg1 and Nrg2 interact physically with the Snf1 kinase. Both proteins interact with the catalytic domain of Snf1 in two-hybrid assays and co-immunoprecipitate with Snf1 from cell extracts.

We show that Nrg2, like Nrg1, functions as a transcriptional repressor. LexA₈₇-Nrg2 represses transcription of a reporter containing LexA binding sites in glucose-grown cells, as previously reported for LexA₈₇-Nrg1 (PARK *et al.* 1999). We also examined the regulation of this repression and found that repression was not relieved by a shift from high to low (0.05%) glucose or by steady-state growth in sucrose, raffinose, or galactose.

Correspondingly, glucoamylase gene expression is not derepressed by growth in these carbon sources (PRETORIUS *et al.* 1986; KARTASHEVA *et al.* 1996). An attempt to assess repression during growth in glycerol plus ethanol, a condition allowing derepression of glucoamylase genes, was inconclusive because the fusion proteins were not expressed well.

The finding that release from repression of *STA* genes occurs only when the carbon source is glycerol/ethanol is at first glance puzzling because the Snf1 kinase is also active during growth on sucrose, raffinose, and galactose. A plausible explanation comes from recent evidence that the Snf1 catalytic subunit and one of the β -subunits of the kinase, Gal83, are enriched in the nucleus when cells are grown on glycerol/ethanol but not when cells are grown on a fermentable carbon source (VINCENT *et al.* 2001). The nuclear localization of Nrg1-GFP is consistent with the idea that nuclear import of Snf1 is required for inhibition of Nrg1 repressor function.

Together, the evidence suggests that Nrg1 and Nrg2 are either direct or indirect targets of Snf1. Previous studies showed that Nrg1 mediates glucose repression of glucoamylase genes (PARK *et al.* 1999) and that Snf1 is required for release from repression (KUCHIN *et al.* 1993). Here we present genetic evidence that Nrg1 and Nrg2 contribute modestly to glucose repression of *DOG2* and that one of the roles of the Snf1 kinase in derepression of this gene is to inhibit repression by Nrg1 and Nrg2. We further show that Nrg1 and Nrg2 interact physically with the Snf1 kinase. Although we did not detect Snf1-dependent phosphorylation of Nrg1 or Nrg2, our data do not exclude the possibility that Snf1 phosphorylates these proteins *in vivo*. Alternatively, other as yet unidentified components may be involved in the regulatory mechanism; for example, Snf1 may phosphorylate another protein that is bound to and affects the function of Nrg1 and Nrg2.

Repression by Nrg1 and Nrg2 may be regulated at multiple steps. However, regulation of Nrg1 function does not appear to involve nuclear export; Nrg1-GFP was localized to the nucleus whether cells were grown in glucose or glycerol plus ethanol. Some control may be exerted at the RNA level. PARK *et al.* (1999) found that *NRG1* RNA levels are 6-fold lower in glycerol/ethanol than in glucose, but it has also been reported that *NRG1* RNA is induced 2.7-fold during the diauxic shift (DERISI *et al.* 1997). *NRG2* RNA is neither significantly induced nor repressed during the diauxic shift (DERISI *et al.* 1997).

The targets and physiological roles of Nrg1 and Nrg2 are still largely unknown, and it is possible that the repressor function of Nrg1 or Nrg2 is regulated in response to other signals besides carbon source. Expression of the *NRG2* gene is clearly regulated by other signals. DNA microarray analysis of genomic expression patterns showed that *NRG2* RNA levels are elevated five-

fold in response to zinc limitation, and *NRG2* has a potential binding site for the zinc-responsive transcription factor Zap1 (LYONS *et al.* 2000). *NRG2* RNA is also induced by alkaline pH, and induction is partially dependent on the transcription factor Rim101, which is required for expression of various alkaline response genes (LAMB *et al.* 2001). The physiological significance of these findings remains to be clarified.

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